Lif1p targets the DNA ligase Lig4p to sites of DNA double-strand breaks

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DNA ligases catalyse the joining of DNA single- and double-strand breaks. *Saccharomyces cerevisiae* Cdc9p is a homologue of mammalian DNA ligase I and is required for DNA replication, recombination and single-strand break repair. The other yeast ligase, Lig4p/Dnl4p, is a homologue of mammalian DNA ligase IV, and functions in the non-homologous end-joining (NHEJ) pathway of DNA double-strand break repair [1–4]. Lig4p interacts with Lif1p, the yeast homologue of the human ligase IV-associated protein, XRCC4 [5]. This interaction takes place through the carboxy-terminal domain of Lig4p and is required for Lig4p stability. We show that the carboxy-terminal interaction region of Lig4p is necessary for NHEJ but, when fused to Cdc9p, is insufficient to confer NHEJ function to Cdc9p. Also, Lif1p stimulates the *in vitro* catalytic activity of Lig4p in adenylation and DNA ligation. Nevertheless, Lig4p is inactive in NHEJ in the absence of Lif1p *in vivo*, even when Lig4p is stably expressed. We show that Lif1p binds DNA *in vitro* and, through *in vivo* cross-linking and chromatin immuno precipitation assays, demonstrate that it targets Lig4p to chromosomal DNA double-strand breaks. Furthermore, this targeting requires another key NHEJ protein, Ku.

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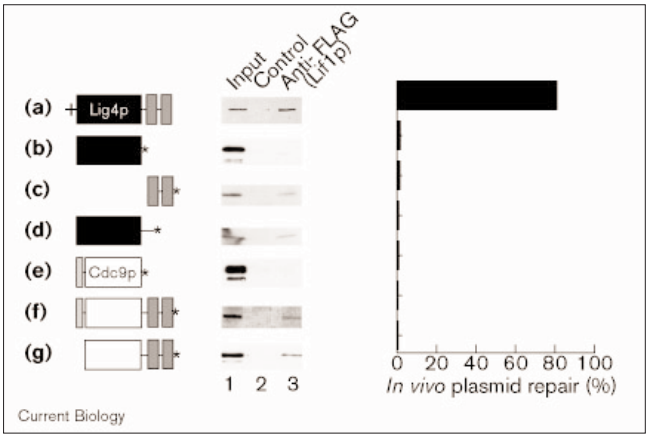
Results and discussion

The carboxy-terminal region of Lig4p/Dnl4p that binds Lif1p contains two motifs that are homologous to the BRCA1 carboxyl terminus [6]. These 'BRCT domains' occur in several DNA repair and DNA damage signalling components, and the BRCT domain of XRCC1 was shown recently to have a compact globular structure [7]. In human DNA ligase IV, the two BRCT domains are separated by a linker region that is necessary and sufficient for interaction with human XRCC4 [8]. To investigate which region(s) of Lig4p interact with Lif1p and are required for NHEJ *in vivo*, we generated a series of epitope-tagged Lig4p derivatives. Immunoprecipitations were performed with anti-FLAG or control antibodies from whole-cell extracts of *lig4* mutant yeast expressing FLAG-epitope-tagged Lif1p and epitope-tagged Lig4p derivatives. The presence of the Lig4p derivative in the immunoprecipitated material was then assessed by Western blotting. Concomitantly, *lig4* mutant yeast expressing the Lig4p derivatives were transformed with linear or supercoiled forms of a yeast–*Escherichia coli* shuttle plasmid, and the number of transformants obtained with linear DNA compared to the number with supercoiled DNA was scored as a measure of *in vivo* NHEJ activity. Figure 1a shows that an epitope-tagged derivative of Lig4p was able to interact with Lif1p (lane 3) and to perform NHEJ efficiently (right panel). Truncation of the entire Lig4p carboxy-terminal region abolished interaction with Lif1p and resulted in a concomitant abrogation of NHEJ activity (Figure 1b). This carboxy-terminal region was sufficient for interaction with Lif1p (Figure 1c). Moreover, a Lig4p fusion protein lacking both BRCT domains, but retaining the inter-BRCT linker region, still interacted with Lif1p (Figure 1d), indicating that, as for human ligase IV [8], the linker region of Lig4p is sufficient for interaction with Lif1p. Strikingly, although the BRCT-deleted form of Lig4p still interacted with Lif1p, its NHEJ activity was abrogated (Figure 1d).

The only other *S. cerevisiae* DNA ligase, Cdc9p, does not have a carboxy-terminal extension like Lig4p, but has an amino-terminal extension that interacts with proliferating cell nuclear antigen (PCNA) [9,10]. Overexpression of Cdc9p does not rescue the NHEJ defect of a *lig4* mutant (Figure 1e). Because the carboxy-terminal region of Lig4p was sufficient for interaction with Lif1p (Figure 1c, lane 3) and contained the BRCT domains necessary for NHEJ, we reasoned that fusion of this region to Cdc9p might confer NHEJ activity on this ligase. Notably, although the resulting fusion protein interacted with Lif1p, it was totally inactive for NHEJ (Figure 1f). To address the possibility that this might be due to a simultaneous interaction with PCNA at replication foci, we constructed a fusion protein lacking the amino-terminal PCNA-interacting region of Cdc9p. This fusion protein interacted with Lif1p but still had no detectable NHEJ activity (Figure 1g). This suggests that, although the carboxy-terminal extension of Lig4p which interacts with Lif1p is necessary for its activity *in vivo*, other differences between Cdc9p and Lig4p also delineate the separate functions of these two DNA ligases *in vivo*.

Yeast and human *lif1* mutant cells have no detectable Lig4p, indicating that Lif1p stabilises Lig4p [5,11]. The

Figure 1

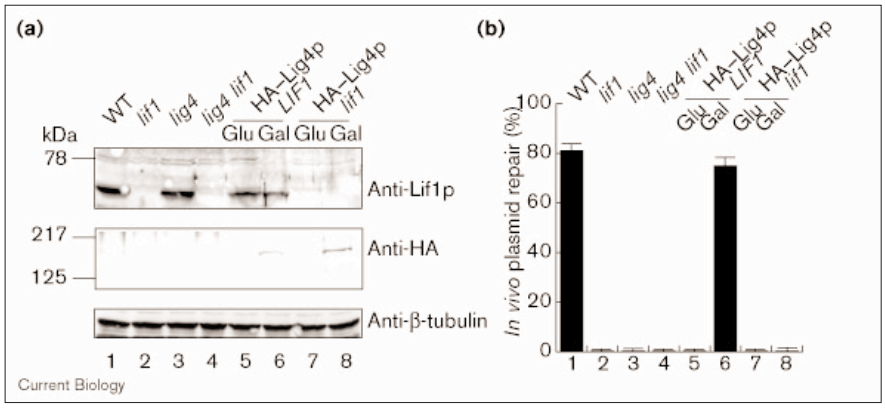


(a–g) Left: Schematic representation of the various galactose-inducible epitope-tagged derivatives of Lig4p or Cdc9p or fusion proteins; plus sign, two copies of the haemagglutinin tag; asterisk, V5 epitope tag; black, Lig4p ligase domain; dark grey, Lig4p BRCT domains; white, Cdc9p ligase domain; light grey, Cdc9p amino-terminal domain. Middle: Proteins were expressed in a *lig4* mutant strain expressing FLAG-epitope-tagged Lif1p. As controls, V5- or HA-tagged Lig4p or Cdc9p fusion proteins were immunoprecipitated with antibodies specific to their epitope tags (lane 1) or an irrelevant monoclonal antibody (lane 2). Interaction with FLAG-tagged Lif1p was assessed by immunoprecipitation using mouse anti-FLAG antibodies (lane 3) followed by western blotting to detect epitope-tagged Lig4p or Cdc9p derivatives. Right: Plasmid repair assays were performed using a *lig4* mutant strain expressing the various fusion proteins. Repair efficiency (expressed as a percentage of that obtained with a wild-type strain) was scored by measuring the number of Leu^+ colonies arising following transformation with *Xba*I-linearised pRS415 versus supercoiled pRS415, and were an average of three independent experiments. Similar results were obtained using *Eco*RI-linearised plasmid pBTM116 (data not shown).

converse appears not to be true, however — western blot analysis using antibodies raised against two different epitopes of Lif1p showed that Lif1p was stably expressed in *lig4* mutant cells (Figure 2a, top panel and data not shown). Neither *lig4* nor *lif1* mutants could perform NHEJ plasmid repair (Figure 2b). The instability of Lig4p in the *lif1* mutant background suggested that the role of Lif1p in NHEJ might simply be to stabilise Lig4p. We therefore reasoned that stable overexpression of Lig4p might rescue the *lif1* mutant phenotype. To test this idea, we constructed a haemagglutinin (HA)-epitope-tagged derivative of Lig4p expressed under the control of the *GAL1–10* promoter. The tagged derivative of Lig4p was expressed at similar levels in the presence or absence of Lif1p (Figure 2a, middle panel, compare lane 6 with lane 8) and was nuclear (data not shown). Notably, whereas overexpression of tagged Lig4p complemented the *lig4* mutant phenotype, its overexpression did not lead to any recovery of *in vivo* plasmid end-joining activity in the *lif1* mutant background (Figure 2b, columns 6,8). These data reveal that, in addition to stabilising Lig4p, Lif1p performs other essential roles in NHEJ.

One other way in which Lif1p might function is by potentiating Lig4p catalytic activity. Indeed, the human Lif1p homologue, XRCC4, stimulates DNA ligase IV activity *in vitro* [12,13]. We therefore expressed and purified from yeast cells HA-tagged Lig4p, either without Lif1p or in a complex with FLAG-epitope-tagged Lif1p, then tested the activity of these proteins *in vitro* in adenylation and DNA ligation assays. DNA ligases form a covalent intermediate with ATP in the absence of DNA that can be readily detected by incubating the protein with [α-^{32}P]ATP. Figure 3a (bottom panel) shows that, in this assay, purified Lig4p formed a stable adenylated protein (lanes 1–3). This adenylation activity was stimulated 2–4-fold by purified recombinant Lif1p (lanes 4–6), to a level similar to that of the purified Lig4p/Lif1p complex (lanes 7–9). To test the activity of purified Lig4p in the ligation of double-stranded DNA, increasing amounts of purified Lig4p, Lig4p with recombinant Lif1p (rLif1p), or the Lig4p/Lif1p complex purified from yeast, were incubated with linearised pBluescript plasmid and the products were analysed on an agarose gel. Lig4p weakly catalysed plasmid DNA multimerisation (Figure 3b, lanes 2–4), regardless of whether the plasmid had 5′ overhangs, 3′ overhangs or blunt ends. The

Figure 2



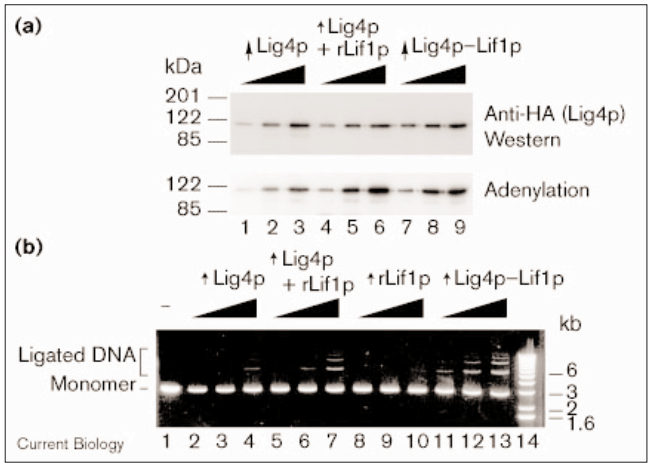
Lif1p is stable in the absence of Lig4p and is essential for NHEJ. **(a)** Approximately 100 μg of whole-cell extracts of the indicated yeast strains were analysed by western blotting using rabbit anti-Lif1p (top), mouse anti-HA (middle; to detect Lig4p) or rat anti-β-tubulin (bottom) antibodies. **(b)** Yeast strains were grown in medium containing glucose (glu; lanes 1–5,7) or galactose (gal; lanes 6,8) and transformed with either supercoiled or *Xba*I-linearised pRS415. Repair efficiency was scored as the number of Leu^+ colonies obtained using linear DNA as a percentage of the number obtained using supercoiled DNA, and were an average of three independent experiments.

activity was greater with 5′ or 3′ overhangs than with blunt ends (data not shown). Addition of Lif1p stimulated this activity 2–4-fold (Figure 3b, lanes 5–7) to a level of activity similar to that of the purified Lig4p/Lif1p complex (lanes 11–13). Lif1p protein itself had no ligase activity (Figure 3b, lanes 8–10) and a non-specific protein (acetylated bovine serum albumin) had no effect on Lig4p activity (data not shown). The fact that the stimulation of DNA ligation is comparable with the stimulation of adenylation activity suggests that Lif1p stimulates Lig4p catalytic activity by increasing the ability of Lig4p to form a pre-adenylated activated complex.

Because Lig4p was significantly active in the absence of Lif1p *in vitro* but did not appear to have any detectable end-joining activity *in vivo*, we reasoned that Lif1p might be necessary for targeting of Lig4p to double-strand breaks within the cell. We therefore tested the ability of Lif1p to bind DNA *in vitro* using the gel retardation assay. Recombinant Lif1p bound co-operatively to a double-stranded DNA molecule of 335 bp in a manner similar to that shown previously for its human homologue XRCC4 (Figure 4a) [13]. However, the apparent selectivity of Lif1p for linear over supercoiled DNA in competition assays was found to be less than that observed with XRCC4 (data not shown) [13]. Moreover, we tested the binding of Lif1p and Lig4p to double-strand breaks *in vivo*. To do this, we induced a single double-strand break in the *MAT* locus in the yeast genome by expressing the HO endonuclease in cells that were mutated for *rad52*. In these cells, homologous recombination is abrogated and cells can repair the double-strand break only via the *LIG4*-dependent NHEJ pathway.

We then performed *in vivo* cross-linking experiments followed by chromatin immunoprecipitations using antibodies against FLAG-tagged Lif1p, HA-tagged Lig4p or endogenous Lif1p (Figure 4b, top, middle and bottom panels, respectively). The *in vivo* localisation of Lif1p or Lig4p to the *MAT* locus was then assessed by subjecting the immunoprecipitates to PCR with *MAT* locus-specific or control primers (see the Supplementary material). Figure 4b (top panel) shows that a PCR product derived from the *MAT* locus was generated from Lif1p immunoprecipitates regardless of whether or not they contained Lig4p. Consistent with this localisation of Lif1p being triggered by the induction of a DNA double-strand break, the PCR product was detected in immunoprecipitates derived from cells that had been grown in the presence of galactose, which leads to HO expression, but not from those of cells grown under HO-repressing conditions (compare lanes 4–6 with lanes 1–3). Immunoprecipitation of Lig4p also retrieved *MAT* locus DNA (Figure 4b, middle panel). Notably, however, this occurred only in the presence but not in the absence of Lif1p (compare lanes 5 and 6, respectively). We also performed experiments with antibodies raised against endogenous Lif1p, with similar results to

Figure 3



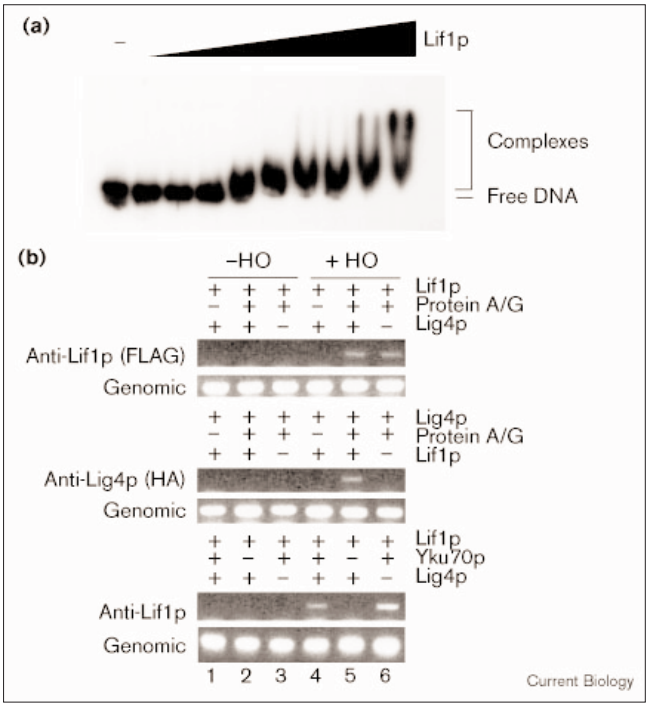
Lif1p stimulates adenylation of and plasmid ligation by Lig4p. **(a)** Lig4p adenylation. Increasing amounts (twofold increments) of purified Lig4p (lanes 1–3), purified Lig4p with a constant excess amount of recombinant Lif1p (rLif1p; lanes 4–6) or purified Lig4p–Lif1p complex (lanes 7–9) were incubated with [α-^{32}P]ATP and analysed by SDS–PAGE. Samples were transferred onto nitrocellulose filters that were then analysed by western blotting using anti-HA antibody (top panel). The blot was then stripped of antibody and exposed to autoradiography to detect radioactively labelled adenylated Lig4p (bottom panel). **(b)** Plasmid ligation. No protein or increasing amounts (twofold increments) of purified Lig4p (lanes 2–4), purified Lig4p with a constant excess amount of rLif1p (lanes 5–7), rLif1p (lanes 8–10) or the purified Lig4p–Lif1p complex (lanes 11–13) were incubated with *Bam*HI-linearised pBluescript (which has a 5′ overhang) and the products were analysed on an agarose gel stained with ethidium bromide.

those obtained with epitope-tagged Lif1p (Figure 4c, bottom panel, lanes 4 and 6). Thus, Lif1p is detected near the DNA double-strand break in the presence or absence of Lig4p, whereas it appears that Lig4p is targeted to such sites only when it is complexed with Lif1p.

Another protein involved in NHEJ is the DNA end-binding protein Ku, and recent work has shown that Ku rapidly associates with DNA double-strand breaks *in vivo* [14,15]. We therefore tested whether the targeting of Lif1p and/or Lig4p to sites of DNA damage *in vivo* might be dependent on Ku. Strikingly, in contrast to the situation in the presence of Ku, where Lif1p is targeted to the *MAT* locus upon HO cleavage (Figure 4c, bottom panel, lanes 4 and 6), Lif1p was no longer targeted to the DNA double-strand break in the absence of Ku (lane 5). Taken together, these results reveal that, although Lif1p is capable of binding linear DNA fragments *in vitro*, its interaction with DNA at the site of a double-strand break *in vivo* is dependent on Ku.

In conclusion, the available data suggest that Lif1p functions in multiple ways to potentiate DNA double-strand break repair by Lig4p. First, Lif1p binds to Lig4p and

Figure 4



Lif1p binds DNA *in vitro* and *in vivo*. **(a)** Gel retardation assays were performed using an α^{32}P-labelled 335 bp DNA fragment and increasing amounts of purified recombinant Lif1p; lanes 1–10 contain 0, 0.5, 1, 2, 4, 6, 8, 10, 16.7 and 20 ng Lif1p, respectively. **(b)** Chromatin immunoprecipitations were performed using yeast whole-cell extracts prepared from strains (see Supplementary material) expressing (lanes 4–6) or not expressing (lanes 1–3) HO endonuclease, and antibodies against FLAG-tagged Lif1p (top), HA-tagged Lig4p (middle), or Lif1p (bottom). PCR reactions were performed on the immunoprecipitated material and on input genomic DNA using oligonucleotides that generated a specific 243 bp fragment derived from a region 400 bp downstream of the HO endonucleolytic site. Control PCR reactions using the PHO5–1 and PHO5–2 primers (see Supplementary material) generated a 518 bp fragment at a distant locus; low levels of this fragment were observed both with or without induction of the HO endonuclease (data not shown). The resulting PCR products were analysed on an agarose gel stained with ethidium bromide.

enhances its intracellular stability [5]. Second, Lif1p stimulates the enzymatic activity of Lig4p, most likely by stabilising the formation of the activated enzyme-adenylate complex. Finally, Lif1p has intrinsic DNA-binding activity and targets Lig4p specifically to sites of double-strand breaks in a manner that depends on functional Ku. One attractive possibility is that the Ku heterodimer directly recruits the Lif1p–Lig4p complex to sites of DNA damage. Alternatively, or in addition, Ku might mediate Lif1p recruitment indirectly, through interacting with an intermediary protein or through triggering alterations in chromatin structure that permit Lif1p access. At least *in vitro*, mammalian Ku has previously been shown to bridge two DNA molecules together [16,17] and this correlates with its ability to stimulate DNA ligation [18]. The availability of biochemical assays for the various NHEJ components, and the ability to assess their interactions with the damaged DNA substrate *in vivo*, should allow the validity of these and other models to be assessed.

Supplementary material

Supplementary material including yeast strains and methodological details is available at http://current-biology.com/supmat/supmatin.htm.

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